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Study of Nephropathia Epidemica in Sweden

Final Report

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The incidence of Nephropathia Epidemica (NE) was determined for various regions of Sweden and compared with the NE antibody prevalence rates in normal population. An incidence of 29 cases per 100.000 population and year was recorded in the most highly endemic area where the antibody prevalence rates exceeded 30% in older age-groups. Approximately 14-20 NE infections per hospitalized case of NE occured for men and women, respectively.
A μ-capture ELISA was developed and proved useful as a rapid diagnostic tool. An IgG ELISA was also developed and compared with IFT. Antigenic relationships between different Hantaviruses were analyzed by IFT and RIPA. Isolates originating from Scandinavia, Belgium and western U.S.S.R. were indistinguishable. Clethrionomys glareolus was found to be the most abundant rodent, as well as the species most frequently found positive. Antibody positive voles were restricted to the northern two thirds of the country, an area corresponding to that where human cases occur.

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SUMMARY

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A μ-capture ELISA was developed and proved useful as a rapid diagnostic tool. An IgG ELISA was also developed and compared with IFT. Antigenic relationships between different Hantaviruses were analyzed by IFT and RIPA. Isolates originating from Scandinavia, Belgium and western U.S.S.R. were indistinguishable. Clethrionomys glareolus was found to be the most abundant rodent, as well as the species most frequently found positive. Antibody positive voles were restricted to the northern two thirds of the country, an area corresponding to that where human cases occur.

FOREWORD

In conducting the research described in this report, the investigator adherd to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator has adherd to policies of applicabel Federal Law 45CFR46.

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TABLE OF CONTENT

Nephropathia epidemica (NE), a member of the Hemorrhagic Fever with Renal Syndrome complex (HFRS), was first described in Sweden by Zetterholm and Myhrman in 1934 (1,2). Until recently, this human disease has been defined only by clinical symptoms (3,4). Clinical criteria for NE disease are: sudden onset of symptoms, elevated body temperature, abdominal or low back pain, gastrointestinal symptoms proteinuria, elevated concentration of serum creatinine, uneventful course, and spontaneous recovery. Although the clinical picture of NE is very dramatic with severe renal dysfunction for several days, no mortality has been recorded.

The etiological agent of NE, Puumala virus (PUU), was isolated in 1983 from a bank vole (Clethrionomys glareolus) (5). This virus isolate made serological confirmation of clinical cases possible and provided a tool for sero-epidemiological surveys.

Primary objectives in the present study:

1. Develope mouse monoclonal antibodies to PUU virus.
2. Develope a rapid diagnostic assay for NE infection in man.
3. Evaluate the rapid diagnostic assay developed in accordance with paragraph 2.
4. Obtain human isolates of PUU virus from clinically ill patients.
5. Determine the clinically apperent attack rate for NE in humans.

Secondary objectives in the present study:

6. Develope human monoclonal antibodies to PUU virus.
7. Determine the anti-PUU virus antibody prevalence rates in humans and in rodents in various regions of Sweden.
8. Continue transmission and pathogenesis studies of PUU virus in Clethrionomys glareolus and other suspected rodent hosts of the virus in nature.
9. Attempt to obtain virus isolates from voles captured in various areas of Sweden.

DEVELOPMENT AND EVALUATION OF RAPID DIAGNOSTIC TESTS FOR NE INFECTION IN MAN

ELISA systems using polyclonal antibodies of different species have been developed and evaluated for detection of human IgM and human IgG antibodies to PUU virus as well as PUU viral antigen.

IgM ELISA

An μ-capture ELISA for detection of human IgM antibodies to PUU virus was employed as follows. Goat anti-human IgM (μ-chain specific; Cappel Laboratories) diluted 1:500 in coating buffer (0.05 M sodium carbonate, pH 9.5-9.7) was absorbed to 96 well polystyrene microtiter plates (Cooke M 29 AR Dynatec laboratories) at 37°C for 2 h. Plates were consecutively treated with human test serum diluted 1:400 in ELISA buffer (PBS without Mg and Ca with 0.05% Tween 20 and 0.5% bovine serum albumin at 37°C for 1 h), virus antigen (undiluted with 0,2% normal human serum at 37°C for 1 h), rabbit anti-PUU virus immunoglobulins (diluted 1:200 in ELISA buffer at 37°C for 1 h), and goat anti rabbit IgG (Kirkegaard and Perry lab) conjugated with alkaline phosphatase (diluted 1:400 in ELISA buffer at 37°C for 1 h). P-nitrophenol-phosphate (Sigma) diluted in diethanolamine buffer (1 M diethanolamine pH 9.8, 0.5 mM MgCl2) was used as substrate. Washing between each step was done 6 times in washing buffer (saline with 0.05% Tween 20).

The reaction was read after 30 minutes at room temperature in a spectrophotometer at 405 nm and expressed as optical density (OD). Optimal dilutions of all reagents used in the ELISA were determined by box titrations.

Antigen was prepared from PUU virus (strain Vindeln 83-223L) infected Vero E-6 cells (CRL 1586; ATCC, Rockville, Md) (5). Cultures were harvested after 20 days. Following low speed centrifugation (2000 RPM for 10 minutes) cells and supernatants were separated. Cell pellets were resuspended in 1/10 of the original volume (using the supernatants as dilutent), sonicated and then centrifuged at 2000 RPM for 10 minutes and the supernatant was used as antigen in the ELISA. A negative control antigen was prepared from uninfected cells by the same procedure.

Immune serum was prepared in a rabbit by use of affinity bead immunization (6,7). A high indirect immunofourescens test (IFT) titer

(one year convalescent) human serum was bound to CnBr activated Sepharose 4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden). The beads were mixed with the antigen mentioned above. Three ml of this preparation mixed with an equal volume of Freund's adjuvant was inoculated into a rabbit by the intramuscular route twice at 6 week-intervals. Serum was collected 9 weeks after the first injection. A purified immunoglobulin preparation was made by use of ammonium sulphate fractionation. Anti human Ig reactive antibodies were eliminated by affinity chromatography using beads identical to those administrated to the rabbit (3,16).

All specimens were tested in duplicate with antigen and negative control antigen. The OD was calculated as the average OD with antigen minus the average OD with negative control antigen. The border between positives and negatives was calculated as the mean of the test result of 48 known negative sera plus 3 standard deviations. An OD of 0.040 or higher was considered positive.

To adjust for plate-to-plate and day-to-day variations in the assay, a positive control serum was included on all plates. This control had an OD of 0.700 (being in the linear interval of this IgM ELISA). If the positive control serum had an OD between 0.500 and 0.900 the plate was accepted: however, all OD values on that plate were multiplied with a factor to set the positive control at 0.700.

Acute, early convalescent and/or late convalescent (3-24 month) sera (n=74) were available from 29 Swedish patients with clinical NE. Sera from patients bled day 5-24 after onset of symptoms were also available from patients with HFRS disease; 13 from European USSR, 9 from Asian USSR and 5 from Korea.

Sera collected during an epidemiological investigation of apparently healthy populations in Sweden, in both HFRS non-endemic (n=48) and endemic areas (n=122), were also tested by IgM ELISA.

Both acute and convalescent sera were available from 24 of the 29 Swedish NE patients investigated. Sera were tested by both IgM ELISA and IgG IFT and the results are seen in table 1. In 15 of the patients the acute sera were collected less than 6 days after onset and 11 (73%) showed a 4-fold or greater titer rise by IFT. When acute sera were drawn 7-14 days post onset of symptoms, 2 (22%) out of 9 revealed a significant titer rise. IgM ELISA results were found positive in all acute and early convalescent specimens.

Late convalescent sera from 8 patients bled at 3-9 months post infection and 10 patients bled at 2 years post-infection, were also tested by IFT and ELISA together with their corresponding acute and/or early convalescent sera (Figure 1 and 2). Most IgM ELISA values were strongly positive in sera collected during the first two months, but at 3-9 months they were in the lower range of significance or negative and at 24 months all sera were negative.

All specimens from patients with clinical NE (n=74) were also tested for rheumatoid factor (Waaler-Rose hemagglutination technique) and all were found negative. All IgM positive sera were confirmed by re-testing after absorbtion with sheep antibodies to human IgG (RF Absorbent; Behringewerke, Marburg, West Germany) (8).

A 24 months convalescent serum (IFT titer 2560) was divided into 3 aliquots and mixed with equal volumes of 3 different RF positive sera (RF titers 1000, 100, 10 respectively) and tested for IgM by ELISA. No positive reactions were seen.

Five sera were tested in parallel in aliquots freeze-thawed once and 6 times, respectively, without any significant difference in OD.

The 122 sera from a normal population in an endemic area were tested for Puumala antibodies by IFT and 19 were found positive. These 19 positive sera were also tested for IgM antibodies by ELISA: 18 were negative and 1 weakly positive with an OD of 0.100.

Acute and early convalescent sera from patients with HFRS disease in the European part of the USSR reacted as NE patients with high IgM ELISA values and high titers to PUU and lower titers to Hantaan virus (HTN) by IFT. Sera from Asian USSR and Korea were weakly positive or negative by IgM ELISA and had very low titers to PUU but high titers to HTN by IFT (Table 2). The IFT data is consistent with the "one way cross" noted in earlier studies (9,10).

An ELISA for detection of specific IgM to HTN virus has been developed by J. Meegan, J. LeDuc and coworkers at the US Army Medical Research Institute of Infectious Diseases. Ten acute sera from NE patients were shipped on dry ice to the USA and tested in the HTN IgM ELISA. All ten sera were found positive by both HTN IgM ELISA and PUU IgM ELISA (data not presented). A serological "one way" cross has previously been well documented between PUU virus and HTN virus (9). This pattern appears to hold true in the IgM assays as well.

IgG ELISA

A sandwich ELISA was employed as follows. Rabbit anti-PUU virus immunoglobulin diluted 1:400 in coating buffer was absorbed to 96 well polystyrene microtiter plates (Cooke M 29 AR Dynatec laboratories) at 37°C for 1 hour, followed by virus antigen (undiluted at 37°C for 1 h), test serum (diluted 1:400 in ELISA buffer at 37°C for 1 h), and swine anti human IgG conjugated with alkaline phosphatase (diluted 1:200 in ELISA buffer at 37°C for 1 h Orion diagnostica, Finland). P-nitrophenol-phosphate (Sigma) diluted in diethanolamine buffer (1 M diethanolamine pH 9.8, 0.5 mM MgCl2) was used as substrate. Washing between each step was done 6 times in washing buffer (saline with 0.05% Tween 20).

The reaction was read after 30 minutes at room temperature in a spectrophotometer at 405 nm and expressed as optical density (OD). Optimal dilutions of all reagents used in the ELISA were determined by box titrations.

All specimens were tested in duplicate with antigen and negative control antigen. The OD was calculated as the average OD with antigen minus the average OD with negative control antigen. The border between positives and negatives was calculated as the mean of the test result of 142 known negative sera (from non-endemic area and negative by IgG IFT) plus 3 standard deviations. An OD of 0.080 or higher was considered positive.

Antigen was prepared from PUU virus (as described for the IgM ELISA). Immune serum was prepared in a rabbit by use of affinity bead immunization (as described for the IgM ELISA).

To adjust for plate-to-plate and day-to-day variations in the assay, a positive control serum was included on all plates. This control had an OD of 0.700 (being in the linear interval of this IgG ELISA). If the positive control serum had an OD between 0.500 and 0.900 the plate was accepted: however, all OD values on that plate were multiplied with a factor to set the positive control at 0.700.

Acute, early convalescent and late convalescent sera (n=49) from 21 Swedish patients with clinical NE were used. Acute sera drawn day 1-9 were available from 18 of these 21 patients, early convalescent (collected day 16-41 post disease onset) from all 21 patients and a late convalescent serum specimen collected 24 months after the acute

infection from 14 of the 21 patients. Sera were also drawn from 5 other patients 12 years following a clinical NE infection.
Sera from patients bled day 5-24 after onset of symptoms were also available from patients with HFRS disease; 13 from European U.S.S.R., 9 from Asian U.S.S.R. and 5 from Korea.
Sera collected during an epidemiological investigation of apparently healthy populations in Sweden, in both HFRS non-endemic (n=142) and endemic areas (n=187), were also tested.
All sera were tested at one dilution by IgG ELISA and titrated for endpoint by IgG IFT. IgG ELISA OD results compared with IgG IFT titers of all 49 sera are seen in figure 3. There was a significant correlation ($r=0,71$ $p < 0,01$) between IgG ELISA OD readings and 10 log IgG IFT titers.
In figure 4, IgG ELISA results from all 21 patients show antibody levels at different times post onset of symptoms. Figure 5 shows IFT results from all 21 patients at different times post onset of symptoms. In the 18 patients with both acute and early convalescent sera only 12 showed a 4-fold or greater titer rise by IFT. As seen in figure 4 and 5 antibody titers and OD increased over time with the highest levels 24 months post infection.
There is no standard way of calculating significant OD increase by ELISA. Different laboratories use different criteria. An OD increase of 100% or more is sometimes used when testing acute and convalescent sera at one dilution. With the limited number of paired sera tested in this study the IgG ELISA does not appear to offer any advatages compared with the IgG IFT.
All 5 sera collected more than 12 years post infection were positive by both assays with ELISA OD between 0.600-0.800 and IFT titers of 64 or more.
All 142 sera from a normal population living in an non-endemic area were tested by IgG ELISA and IgG IFT and found negative. Out of 187 sera tested by IgG IFT from endemic area 34 were found positive with a titer of 32 or greater. Identical results were found with IgG ELISA. All ELISA positive sera had an OD of 0.200 or greater.
Sera from U.S.S.R. and Korea were all tested by IgG ELISA and IgG IFT using both PUU virus and HTN virus as antigen. Acute and early convalescent sera from patients with HFRS disease in the European part of the U.S.S.R. reacted as NE patients with high IgG ELISA values and

high titers to PUU and lower titers to HTN by IgG IFT. Sera from Asian U.S.S.R. and Korea were weakly positive or negative by IgG ELISA and had very low titers to PUU but high titers to HTN by IFT (Table 3).

Antigen ELISA

ELISA for detection of PUU virus antigen has also been developed and evaluated. Several formats have been tried for antigen detection. The assay giving the highest sensitivity and specificity includes the use of 2 different late convalescent human sera with very high titers by IFT. Purified immunoglobulin preparations of the 2 human sera were made by use of ammonium sulphate fractionation. In this format plates were coated with a human capture antibody (diluted 1:400 in coating buffrer), specimen added and specific PUU antigen indicated by a peroxidase conjugated human serum (diluted 1:250 in ELISA buffer). The system has been evaluated using both cell culture grown virus and virus in lung suspensions from infected C. glareolus. Wild C. glareolus trapped in both endemic and non-endemic areas of Sweden as well as bank voles infected in the laboratory have been tested for presence of PUU virus antigen in lung tissue using both both IFT (cryostat-sections) and ELISA (lung suspension). The number of specimens in this comparison have been limited but the results show a very good agreement. All IFT positive samples were also positive by ELISA. A few antigen IFT negative samples were positive for antigen by ELISA. These bank voles were all antibody positive and are therefore considered true positives.

In Vero E6 cell infected supernatants, approximately 100 TCID50 can be detected by the antigen ELISA.

Twenty sera from acutely ill NE patients (collected between day 1-10) have been tested undiluted and diluted 1:10 for presense of PUU antigen with negative results. This test is presently being developed further.

ANTIGENIC RELATIONSHIPS BETWEEN HANTAVIRUSES ANALYZED BY IMMUNOPRECIPITATION

Antigenic relationships between seven hantaviruses isolated in Sweden, Belgium, Korea, European U.S.S.R. and Asian U.S.S.R. were studied by radioimmune precipitation assay (RIPA) and IFT. Seven strains of hantaviruses were used. Strains 76-118 (Hantaan) and 80-39 (Urban rat) were isolated in Korea; 83-223L (Puumala virus) in Sweden; CG-13891 in Belgium; and CG-1820, K-27 and 4605 in the U.S.S.R. Their origin and isolation sites are summarised in Table 4. All strains were propagated in Vero E6 cells maintained in Eagles MEM supplemented with 2% fetal bovine serum.

Paired sera (acute and early convalescent) were available from 10 Swedish patients with clinical NE.

Sera from patients bled day 5-24 after onset of symptoms were also available from 15 patients with HFRS disease from European U.S.S.R., 9 from Asian U.S.S.R. and 5 from Korea. Late convalescent sera (collected 2-12 months after the infection) were available from 2 patients from Sweden, 1 patient from European U.S.S.R., 2 patients from Asian U.S.S.R. and 1 patient from Korea.

Sera from 2 C. glareolus infected in the laboratory with 83-223L and bled 50 days post inoculation were also tested.

Immune serum to 83-233L strain was prepared in a rabbit using affinity bead immunization (as described for the IgM ELISA).

Immune serum to prototype Hantaan strain (76-118) and Urban rat (80-39) was also prepared using a single dose injection of Vero E6 grown virus. Serum was collected 8 weeks post inoculation.

Fourteen days post-infection, the virus infected Vero E6 cells (150cm2 confluent monolayer) were prepared for radiolabelling by incubation with Eagle's MEM containing Actinomycin D (10µg/ml) for 3h at 37°C. The cell monolayer was then washed with PBS; 20ml of Eagles MEM containing one-tenth the normal concentration of methionine, 1mCi [35S]methionine, 2% fetal bovine serum and 10µg/ml Actinomycin D added; and reincubated at 37°C for 36h. The supernatant medium was removed; clarified; and the virus pelleted by centrifugation at 85000g for 75min. For the 76-118, 80-39 and 4605 strains, the virus pellet was resuspended in 3ml RIPA buffer: 2% Triton X-100, 0.7M KCl, 0.15m NaCl, 1% Trasylol Aprotinin, 0.01M Tris-HCl pH 7.8; and sonicated. The

suspension was kept at 4°C for 1h and centrifuged at 14000g for 30min. The supernatant was used for immunoprecipitation. For the 83-223L, CG-1820, CG-13891 and K-27 strains, the close migration on SDS-PAGE of the G2 and N proteins made their analysis by immunoprecipitation difficult. Consequently, the antigens for these strains were processed to a glycoprotein and a nucleocapsid enriched fraction, and each serum then simultaneously tested against both fractions. The pelleted virus was resuspended in RIPA buffer without KCl. The suspension was kept at 4°C for 1h and centrifuged at 14000g for 30min. The supernatant was equally divided and each lot layered onto a sucrose cushion consisting of 0.15ml 60% (w/w) sucrose with 0.65ml 15% (w/w) sucrose on top. Samples were spun in a SW60 rotor. For the glycoprotein fraction, centrifugation was performed at 50000rpm for 175min. The supernatant above the sucrose cushion was saved and made 0.7M with solid KCl. For the nucleocapsid enriched fraction, centrifugation was performed at 50000rpm for 135min. The supernatant was removed and the sucrose fraction made upto the same volume as the glycoprotein fraction with RIPA buffer without KCl and finally made 0.7M with solid KCl.

RIPA samples were analysed by discontinuous slab SDS-PAGE as previously described. The spacer gel consisted of 4.5% (w/v) acrylamide and 0.12% (w/v) N,N-methylenebisacrylamide (bis) and the separation gel consisted of 12.5% (w/v) acrylamide and 0.34 % (w/v) bis. Gels were fixed in a solution of 10% (v/v) glacial acetic acid and 30% (v/v) methanol, processed for fluorography using "Enlightening," and dried and exposed to Kodak X-Omat AR-5 films at minus 70°C.

The two envelope glycoproteins G1 and G2, and the nucleocapsid (N) protein could be readily identified in RIPA for all strains examined (exemplified in figure 6). The fourth viral structural protein, the large protein, could not be efficiently immunoprecipitated and is therefore not considered further in this study. No major mol. wt. differences were seen between the strains examined in their G1 (72-74K) and G2 (55-57K) glycoproteins. However, the N protein of strains 76-118, 80-39 and 4605 had mol. wt. of 50K, whereas that of strains 83-223L, CG-13891, CG-1820 and K-27 had mol. wt. of 54K.

RIPA analysis of sera from 20 Swedish NE patients was carried out using the "homologous" 83-223L strain antigen. Most of the acute phase sera, although showing high titers in IFT, only precipitated the N

protein. The early convalescent phase sera precipitated the N protein and the G1 and G2 proteins poorly. The late convalescent phase sera precipitated all three viral proteins strongly. RIPA analysis of 6 Korean KHF patient sera using the homologous 76-118 strain gave similar results. The five early convalescent phase sera precipitated only the N protein, while the one late convalescent phase serum available also precipitated the G1 and G2 proteins. Similar data were obtained for the U.S.S.R. patient sera. Therefore, late convalescent phase sera were preferentially selected for cross-strain RIPA analyses.

The cross-strain RIPA data with the selected HFRS patient sera are tabulated in Table 5 and exemplified in Figure 6. The cross-IFT data for the same sera are also included in Table 5. In terms of overall degree of cross-reactivity, the IFT and RIPA data always matched. The Swedish, Belgian and the two European U.S.S.R. virus isolates exhibited the same patterns of reactivity (designated NE-type strains). The N, G1 and G2 proteins of these strains were strongly immunoprecipitated by the Swedish NE patient and European U.S.S.R. patient sera. RIPA of these strains using the Asian U.S.S.R. and Korean sera were essentially negative. The Korean (76-118) and Asian U.S.S.R. (4605), designated KHF-type virus isolates, were seen to be antigenically very closely related to each other but distinct from the NE-type isolates. The IFT data for the patient sera indicate the presence of a one way cross-reaction between these two antigenic groups. This is borne out in RIPA. Whereas the KHF-type patient immune sera failed to react with the NE-type virus protiens, the N-protein of the KHF-type virus isolates was distinctly and the G1 and G2 proteins weakly immunoprecipitated by the NE-type patient sera. The Urban rat isolate (80-39), appeared to be antigenically related in all three viral proteins to both the above two antigenic groups. However, its pattern of reactivity with the different patient sera indicated that it was distinct from isolates of either of the two groups.

Cross-RIPA and cross-IFT using animal immune sera are tabulated in Table 6 and a RIPA exemplified in Figure 6. The antigenic groupings seen in cross-analyses with patient sera were also seen with the animal sera. However, instead of the one way cross-reaction between the NE-type group and the KHF-type group seen with patient sera, a reciprocal cross-reaction between the two groups was seen with the

rabbit immune sera in both RIPA and IFT. The N protein was seen to be the major cross-reactive antigen between the two groups of isolates. Of the two glycoproteins, the G2 protein exhibited weak cross-reactivity and the G1 protein no cross-reactivity between the two groups of isolates. The Urban rat (80-39) isolate was again seen to be antigenically related to and yet distinct from the other two groups of isolates. The Urban rat isolate's N protein was the major and the G2 glycoprotein the minor cross-reactive antigen between the groups. There was no cross-reactivity between G1 glycoproteins of the NE-type and Urban rat isolates. Similarly, rabbit anti-Urban rat serum failed to precipitate the G1 of the 76-118 and 4605. However the rabbit anti-76-118 serum did give weak precipitation of the Urban rat G1 protein. In addition to the rabbit immune serum, sera from C. glareolus experimentally infected with the 83-223L isolate were also examined. These vole sera showed the same patterns of cross-reactivity as did the rabbit anti-83-223L.

DEVELOPMENT OF MOUSE AND HUMAN MONOCLONAL ANTIBODIES

Attempts to develop mouse monoclonal antibodies have been made at 3 different occasions. The experiments have been performed in collaboration with investigators from the virological department of the Karolinska Institute who are familiar with the techniques. Initially attempts were made to adapt PUU virus to suckling mice. As many as 8 blind passages with both cell culture grown PUU virus and infectious lung material from C. glareolus were used several times without success. Mice have also been immunized using affinity bead immunization (as described for rabbit immunization in IgM ELISA). A virus preparation purified by ultracentrifugation followed by sucrose-gradients has also been tried as an immunogen as well as supernatants of infected Vero E6 cells and sonicated cell lysates. Mice were tested by IFT and ELISA for antibody response and all had no or low titers to PUU virus. Lately it has come to our attention that the mouse colony used in our laboratory is infected with mouse hepatitis virus. The chief veterinarian at Karolinska Insitute claims that several groups making hybridomas earlier without problems now fail to immunize mice in certain systems while other systems work as before. If mouse hepatitis virus is the reason for our failure or if difficulties in immunizing mice with PUU virus have other reasons is not clear. Production of human monoclonals has not yet been attempted.

ATTEMPTS TO OBTAIN PUU VIRUS ISOLATES FROM VOLES AND FROM CLINICALLY ILL PATIENTS

All wild rodents trapped between 1982-1984 and found PUU antigen positive in lung-section have been processed for virus isolation. Lung material was inoculated into non-infected colonized C. glareolus. Lungs were harvested from animals which seroconverted and cocultivated with Vero E6 cells following the procedure described in reference 5. Three strains of PUU virus have been isolated and made available to USAMRRID. All 3 "new" strains originats from C. glareolus trapped at the same location (Hällnäs) where the origanal Swedish PUU strain was recovered.

Attempts to isolate PUU virus from human has been done following several procedures;

a). Twenty sera drawn between day 1-4 post onset of disease have been inoculated onto Vero E6 cells and passaged using standard procedure (5).

b). All sera were also inoculated into uninfected (colonized) C. glareolus without any animals seroconverting.

c). Ten blood specimens from NE patients bled during the first week of disease where sent to SBL were lymphocytes were separated, stimulated and then cocultivated with Vero E6 cells for 5 weeks.

No human HFRS virus has been recovered.

EPIDEMIOLOGY OF NE IN SWEDEN. ANTIBODYPREVALENCE RATES IN HUMANS AND IN SMALL MAMMALS. ATTACK RATE IN HUMANS.

Human sero-survey.

Regional health facilities were requested to obtain 100-250 human sera from out patient volounteers > 10 years of age, independently of these persons previous history of infectious disease. Human sera were obtained from 19 communities representing nine counties in Sweden. The names of the cummunities are seen in figure 7A. A total of 2.534 sera were examined by IgG IFT using PUU virus as antigen. Of these 202 (8%) possesed antibodies. Location, number of sera, age distribution and results are seen in table 7 and 8 and figure 7A and 7B. The highest antibody prevalence rates were found in Västerbotten county, intermediate rates were found in the central counties and the far north, and the lowest rates were found in the southern counties (Malmö and Gotland).

Incidence of NE in Sweden.

All serologically confirmed NE cases in Sweden are reported to the Department of Epidemiology at SBL. All cases recorded between 1 June 1984 and 31 May 1985 were used to calculate incidence rates. The period represents a period with high numbers of small rodents in the endemic region. The highest incidence rates were seen in Västerbotten and Västernorrland counties, with rates of 29,4 and 20,8 cases per 100.000 population, respectively. In Jämtland and Norrbotten counties, incidence rates were about one-half of those of Västerbotten and Västernorrland counties (12,6 and 12,1 per 100.000 population, respectively). The rates in Värmland, Kopparberg, and Gävleborg counties were substatially lower than those in the north and ranged from 5,0 to 7,0 per 100.000 population. Incidence rates for the remainder of the counties, all located in the southern one-third of Sweden, were < 0.8 per 100.000 population. Incidence rates by county for the 1984-1985 season are shown in figure 7C and table 9.

The incidence of NE was also compared with the antibody prevalence in a highly endemic area of Västerbotten county. As part of an earlier study, hospital files from the departments of medicine, infectious

diseases and pediatrics in Västerbotten county were investigated for NE cases (4). Information on age, sex, and area of residence were collected between December 1959 and April 1974 for all cases fullfilling the clinical criteria of NE. Three municipal districts in Västerbotten county (Vindeln, Sorsele and Norsjö) were selected for this study (see figure 7A). The population of these 3 areas were divided into sex and age-groups and the number of residents was calculated as the mean for census years 1960, 1965, 1970, 1975. Calculation of the accumulated risk to contract NE during a life-time (seven decades) was based on age specific annual incidence rates for each age group. The annual incidence was multiplied by 10 to cover a decade and incidences per decade were added together.

The mean number of inhabitants in Vindeln, Norsjö and Sorsele (between 1960 - 1975) and the number of clinical NE cases (December 1959 - April 1974) are shown in table 10. Puumala virus specific antibodies by IFA are also shown in the table 10. Antibody prevalence rates increased with age and reached 40% in males and 15% in females in the age group of 60 years or older. The male:female ratio for the accumulated risk of contracting NE over a life time was 3,7:1. This is comparable with the sex-ratio of antibody prevalence 2,6:1 in the age group 60 years or older.

The chance of being hospitalized with NE during life-time in this area is 2,9% for men and 0,8% for women. To compare this frequency with the antibody prevalence rates in the oldest age groups we must assume that NE infection does not affect the mortality in the population and that NE infection induces lifelong persisting antibodies detectable by IFA. Furthermore, we must assume that the endemic situation has been constant when comparing clinical attack rates as recorded during the time period 1959-1974 with results of serology performed 1985 when sera were collected. Since antibody prevalence rate in the oldest age group are 40% for men and 15% for women, this indicates that there are 14 and 20 NE infections per hospitalized case of NE for men and women, respectively. If the assumptions that NE infection does not affect population mortality and/or that NE antibodies persist for life are incorrect, the ratio of infections per hospitalized case will increase.

In the previous survey we found 243 serologically confirmed cases were recorded during a year when the vector (C. glareolus) was prevalent in

the endemic region of Sweden. Extrapolating from the ratio of infections per recorded cases this would mean approximately 4.000 infections during that year. These cases occured in a population of 2.300.000 living in the endemic area of Sweden. Although the calculations made above are based on approximations and not intended to be exact they indicate the magnitude of NE infections.
It remains to be determined whether the clinical presentations in cases not getting medical attention are typical, atypical or mild or if some cases of NE are asymptomatic. Further details of this study are given in reference 11.

Small mammals

Small mammals trapped live at different locations in Sweden were tested for presence of Puumala virus specific antibodies in serum and PUU virus specific antigen in lung sections. Antibodies in serum were tested by IFT using FITC conjugated rabbit anti C. glareolus Ig (L. R. Bagley USAMRIID) for Clethrionomys sera and sheep anti mouse Ig (SBL) for Apodemus sera. Detection of PUU virus antigens in lung tissues was done by cryostat section fixed in acetone and stained by IFT as described in reference 12.

682 small mammals were captured at eight geographically separate sites during 1982-1984 (Figure 7A). The species most frequently captured was C glareolus (407), followed by C. rufocanus (125) and A. sylvaticus (64). Both PUU virus antibodies and PUU virus antigen were most frequently found in C. glareolus; however, antibody to PUU virus, but not antigen, was found in single specimens of C. rufocanus, Apodemus flavicollis, and Rattus norvegicus (Table 11).)

The highest antibody prevalence rates were detected in Sundsvall area and around Vindeln and were 35% and 17% respectively. Antigen prevalence rates were approximately one-half the antibody prevalence rates, or 18% and 10% respectively. Only one single C. glareolus south of Limes Norrlandicus was found to have antibody to PUU virus.

Vole colonies of C. glareolus from both endemic and non-endemic areas, C. rufocanus and C. rutilus have been established in the laboratory. Three different subspecies of C. glareolus are recognized and animals from all three have been colonized. One variant of C. glareolus from a non-endemic area (south of Limes Norrlandicus) and 2 from endemic areas north of Limes Norrlandicus have been investigated.

In earlier experiments cellculture grown PUU virus was compared with lung material from infected C. glareolus. Cell culture grown virus and lung material were titrated using 10 fold dilutions in C. glareolus. Animals were then tested for presence of PUU specific antigen in lungs and antibodies in serum. Animals infected with cell culture material did not have detectable antigen in their lungs while animals receiving lung material and seroconverted most often also reveiled antigen in their lung specimens. A pool of lungs from infected C. glareolus was therefore prepared and used in a comparison of susceptibility between

different species. All species compared in this experiment received 100 ID50 (determined in C. glareolus). Animals were inoculated subcutaneously and sacrificed 60 days later. Sera were tested for presence of PUU specific antibodies and lungs were tested for antigen by ELISA as described earlier. All animals were also tested for presence of antibodies to PUU virus, HTN virus and Urban rat virus before being inoculated and all were found negative.

Wild trapped Mus musculus (n=10) and Apodemus flavicollis (n=10) were similarily investigated and all found negative (table 12.). C. glareolus, C. rufocanus and C. rutilus were all colonized. The number of animals in the experiment and the results are seen in table 12. The results show that C. glareolus from both endemic and non-endemic area are susceptable to PUU infection but that animals from highly endemic areas had higher rates of antigen positives. C. rutilus and C. rufocanus both originating from endemic areas have lower susceptibility to the virus. The number of animals included in this experiment was very low for some species and the experiment is continuing. Animals will also be tested for infectivity. Since PUU virus can not be assayed in cell culture, infectivity will be assayed in C. glereolus (Ammar).

Table 1 Acute and early convalescent sera from 24 Swedish patients with clinical NE bled at different intervals post onset of symptoms and tested by IgM ELISA and IgG IFT. OD values are multiplied by 1000 and a value of 40 or higher is considered positive.
* = 4-fold or greater titer-rise against PUU virus by IFT.

PATIENT NR	DAYS AFTER ONSET OF DISEASE	IgM ELISA OD 405 nm	IFT TITERS	
1	1	86	8	*
	32	171	1024	
2	2	677	1024	
	22	553	2048	
3	2	597	64	*
	31	182	1024	
4	2	552	128	*
	41	291	1024	
5	3	607	128	*
	30	595	1024	
6	4	407	1024	
	20	178	2048	
7	4	600	16	*
	22	456	512	
8	4	309	256	*
	31	121	1024	
9	4	433	32	*
	38	674	1024	
10	5	496	32	*
	16	228	1024	
11	5	207	128	*
	19	116	2048	

Table 1 (cont.)

12	5	379	128	
	20	400	256	
13	5	478	128	*
	36	315	1024	
14	6	653	256	*
	18	483	1024	
15	6	467	256	
	33	118	512	
16	8	281	512	*
	28	178	2048	
17	8	140	1024	
	23	72	512	
18	8	659	512	
	15	470	256	
19	9	542	1024	
	29	220	1024	
20	11	671	256	*
	38	83	1024	
21	12	824	256	
	23	808	256	
22	13	543	1024	
	29	260	1024	
23	13	691	4096	
	35	246	2048	
24	14	700	256	
	40	629	128	

Table 2. Acute or early convalescent sera from HFRS patients collected in different geographic regions (European USSR, Asian USSR and Korea) and tested by IgM ELISA, IgG IFT using PUU virus and HTN virus as antigens. OD values are multiplied by 1000 and a value of 40 or higher is considered positive.

Sera no	Days post onset	ELISA IgM	IFT (PUU)	IFT (HTN)
Asian USSR				
1.	9	0	32	8192
2.	7	34	16	8192
3.	9	28	32	8192
4.	8	22	64	8192
5.	10	2	64	2048
6.	9	58	128	8192
7.	9	54	32	8192
8.	5	1	< 8	8192
9.	10	0	1280	>32768
Korea				
10.		60	8	2048
11.		70	8	1024
12.		28	16	2048
13.		45	16	64
14.		35	8	1024

Table 2 (cont.)

Sera no	Days post onset	ELISA IgM	IFT (PUU)	IFT (HTN)
European USSR				
15.	12	586	2048	512
16.	20	281	8192	256
17.	15	520	2048	128
18.	13	460	2048	512
19.	10	475	2048	128
20.	18	787	2048	512
21.	14	948	2048	512
22.	5	388	2048	64
23.	13	259	2048	256
24.	18	488	2048	64
25.	16	687	8192	512
26.	19	442	2048	256
27.	14	545	2048	256

Table 3.

Acute or early convalescent sera from HFRS patients collected in different geographic regions (European U.S.S.R., Asian U.S.S.R. and Korea) and tested by IgG ELISA, IgG IFT using PUU virus and HTN virus as antigens. OD values are multiplied by 1000 and a value of 80 or higher is considered positive.

Acute, early convalescent and 2 years sera from seven Swedish patients are added for comparecy.

Sera no	Days post onset	ELISA IgG	IFT (PUU)	IFT (HTN)
Asian U.S.S.R.				
1.	9	7	32	8192
2.	7	23	16	8192
3.	9	4	32	8192
4.	8	65	64	8192
5.	10	18	64	2048
6.	9	0	128	8192
7.	9	32	32	8192
8.	5	16	< 8	8192
9.	10	0	1280	>32768
Korea				
10.		7	8	2048
11.		3	8	1024
12.		0	16	2048
13.		183	16	64
14.		13	8	1024

Table 3 (cont.)

Sera no	Days post onset	ELISA IgG	IFT (PUU)	IFT (HTN)
European U.S.S.R.				
15.	12	359	2048	512
16.	20	411	8192	256
17.	15	330	2048	128
18.	13	405	2048	512
19.	10	180	2048	128
20.	18	436	2048	512
21.	14	503	2048	512
22.	5	49	2048	64
23.	13	224	2048	256
24.	18	264	2048	64
25.	16	357	8192	512
26.	19	361	2048	256
27.	14	487	2048	256

Table 4.

History of HFRS strains employed in this study

Virus strain	Isolated from: Animal host	Location
Puumala 83-223L	Clethrionomys glareolus	Sweden
CG-13891	Clethrionomys glareolus	Belgium
CG-1820	Clethrionomys glareolus	USSR (European part)
K-27	HFRS patient	USSR (European part)
Hantaan 76-118	Apodemus agrarius	Korea
4605	HFRS patient	USSR (Asian)
Urban rat 80-39	Rattus norvegicus	Korea

TABLE 5. Reactivity of HFRS patient sera with hantavirus strains in IFT[1] and RIPA[2].

	VIRUS STRAIN														
	83-223L				CG-13891[3]	CG-1820[3]	K-27[3]	76-118				4605[4]	80-39			
	IFT	RIPA			IFT	IFT	IFT	IFT	RIPA			IFT	IFT	RIPA		
Patient sera[5]		G1	G2	N					G1	G2	N			G1	G2	N
European USSR patient T	512	+	+	+	2048	2048	2048	512	$+^{W}$	$+^{W}$	+	2048	512	+	+	$+^{W}$
European USSR patient 3258	8192	$+^{W}$	$+^{W}$	+	2048	8192	2048	512	-	-	+	512	512	-	-	+
Swedish NE patient M	2048	+	+	+	2048	2048	8192	2048	$+^{W}$	$+^{W}$	+	512	512	+	+	$+^{W}$
Swedish NE patient RG	2048	+	+	+	2048	512	2048	512	$+^{W}$	$+^{W}$	+	512	128	+	$+^{W}$	+
Asian USSR patient 27-2	32	-	-	-	8	32	8	512	+	+	+	512	512	$+^{W}$	$+^{W}$	+
Asian USSR patient 83-3	32	-	-	-[6]	32	32	32	512	+	+	+	2048	512	$+^{W}$	+	+
KHF (Korea) patient 700047	8	-	-	-	8	8	8	512	+	+	+	512	512	-	+	+

[1] IFT titers are expressed as the reciprocal of the highest serum dilution which exhibited detectable reactivity.

[2] RIPA reactivity is expressed as: + distinct; $+^{W}$ weak and - none for the viral G1, G2 and N proteins.

[3] RIPA reactivity was the same as shown for strain 83-223L.

[4] RIPA reactivity was the same as shown for strain 76-118.

[5] All sera were late convalescent (2 months up to >2 years) except for European USSR patient 3258 serum which was taken 16 days upon disease onset.

[6] A very weak immunoprecipitation of the N protein was seen.

TABLE 6. Reactivity of rabbit sera with hantavirus strains in IFT[1] and RIPA[2].

	VIRUS STRAIN														
	83-223L				CG-13891[3]	CG-1820[3]	K-27[3]	76-118				4605[4]	80-39			
	IFT	RIPA			IFT	IFT	IFT	IFT	RIPA			IFT	IFT	RIPA		
Rabbit immune sera[5]		G1	G2	N					G1	G2	N			G1	G2	N
Anti-83-223L virus	512	+	+	+	512	512	512	128	-	+[W]	+[W]	128	8	-	+[W]	+
Anti-80-39 virus	128	-	+[W]	+	128	128	128	512	-	+[W]	+	512	2048	+	+	+
Anti-76-118 virus	32	-	+[W]	+	32	32	32	128	+	+	+	128	32	+[W]	+	+
83-223L virus infected vole sera[5]																
1	128	+	+	+	NT	NT	NT	NT	-	+[W]	+	NT	NT	-	+[W]	+
2	128	+	+	+	32	32	8	<8	-	-	+[W]	8	<8	-	-	+

[1-4] see footnotes Table 5.

[5]Serum collected 60 days post-infection.

NT = not tested.

Table 7. Antibody prevalence to PUU virus in normal population tested by IFT. Locations are seen on figure 7A.
M=male F=female

Age	Kiruna(BD)		Vittangi(BD)		Gällivare(BD)		Haparanda(BD)	
	M	F	M	F	M	F	M	F
5-9	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1
10-14	0/0	0/0	0/0	0/1	0/0	0/0	0/2	0/3
15-19	0/3	1/7	0/0	0/6	0/3	0/0	0/2	0/4
20-29	0/8	0/8	0/5	0/6	0/5	0/15	0/7	0/15
30-39	1/8	1/10	0/2	0/13	0/8	2/14	1/20	2/18
40-49	0/9	0/13	1/3	0/13	3/6	1/10	0/1	0/13
50-59	1/12	0/17	3/18	1/11	1/13	2/8	0/4	0/6
60-69	1/9	0/5	2/17	1/9	1/8	1/9	1/8	3/13
> 70	0/7	0/9	1/5	1/3	1/4	0/9	1/4	0/10
total	3/56	2/69	7/50	3/62	6/47	6/65	3/48	5/83

Age	Vindeln(AC)		Norsjö(AC)		Sorsele(AC)	
	M	F	M	F	M	F
5-9	0/0	0/0	0/0	0/0	0/0	0/0
10-14	0/2	0/1	0/0	0/0	0/1	0/0
15-19	0/2	0/2	1/1	0/1	0/0	0/3
20-29	0/11	0/33	1/4	0/5	0/1	1/9
30-39	2/12	1/22	1/3	0/3	0/5	1/4
40-49	0/9	0/10	1/3	0/9	1/3	1/12
50-59	2/12	6/18	0/6	3/16	1/7	1/7
60-69	11/25	1/25	3/11	4/12	8/16	1/4
> 70	15/39	4/25	3/5	2/11	4/15	2/14
total	30/112	12/136	10/33	9/57	14/48	7/53

Table 7 (cont).

Age	Fränsta(Y)		Stöde(Y)		Kvissleby(Y)	
	M	F	M	F	M	F
5-9	0/0	0/0	0/0	0/0	0/0	0/0
10-14	0/0	0/0	0/0	0/0	0/0	0/0
15-19	0/1	0/0	0/2	0/2	0/0	0/1
20-29	0/1	0/0	2/4	0/4	0/6	0/2
30-39	0/3	0/3	1/5	0/11	0/9	1/5
40-49	0/2	1/4	0/3	0/8	1/8	0/2
50-59	1/8	0/6	3/9	0/4	0/8	0/4
60-69	4/13	1/14	3/9	3/12	1/10	0/12
> 70	1/19	0/22	2/8	3/17	3/11	1/17
total	6/47	2/49	11/40	6/58	5/52	2/43

Age	Rättvik(W)		Edsbyn(X)		Bollnäs(X)	
	M	F	M	F	M	F
5-9	0/0	0/0	0/0	0/0	0/0	0/0
10-14	0/0	0/0	0/0	0/0	0/0	0/0
15-19	0/0	0/0	0/4	0/2	0/0	0/2
20-29	0/1	0/3	0/13	0/8	0/0	0/4
30-39	0/4	0/6	2/12	1/13	0/5	0/2
40-49	0/5	0/6	0/3	0/3	0/0	0/9
50-59	1/4	0/14	0/8	1/5	0/4	0/7
60-69	1/8	1/15	5/14	0/16	2/9	1/17
> 70	1/12	1/16	5/21	2/21	0/8	0/23
total	3/34	2/60	12/75	4/68	2/26	1/64

Table 7 (cont).

Age	Fagersta(U)		Torsby(S)		Arvika(S)	
	M	F	M	F	M	F
5-9	0/0	0/0	0/0	0/0	0/0	0/0
10-14	0/0	0/3	0/0	0/0	0/0	0/0
15-19	0/2	0/5	0/3	0/4	0/2	0/2
20-29	0/7	0/4	1/11	1/9	1/8	1/28
30-39	1/13	2/14	0/9	0/16	0/13	0/26
40-49	1/6	1/12	0/5	1/19	0/5	0/17
50/59	2/12	0/9	2/12	0/21	1/12	1/15
60-69	0/6	0/3	2/22	3/37	0/8	2/9
> 70	0/2	0/1	1/14	2/18	1/6	0/10
total	4/48	3/51	6/76	7/124	3/54	4/107

Age	Lund(M blood-donors)		Gotland(I)		Dalby/Lund(M)	
	M	F	M	F	M	F
5-9	0/0	0/0	0/3	0/1	0/0	0/0
10-14	0/0	0/0	0/6	0/3	0/0	0/3
15-19	0/28	0/0	0/2	0/2	0/6	0/4
20-29	1/60	0/21	0/4	0/7	0/8	0/23
30-39	0/37	0/13	0/11	0/6	0/25	0/34
40/49	0/24	1/6	0/6	0/5	0/14	0/17
50-59	0/8	0/2	0/4	0/8	0/3	0/7
60-69	0/0	0/0	0/13	0/19	0/1	0/3
> 70	0/0	0/0	0/43	0/40	0/9	0/5
total	1/157	1/42	0/92	0/91	0/66	0/96

Table 8. Antibody prevalence to PUU virus in normal population tested by IFT. Locations are seen on figure 7A. Results are presented for each county. M=male F=female

Age	County BD		County AC	
	M	F	M	F
5-9	0/0 (0%)	0/1 (0%)	0/0 (0%)	0/0 (0%)
10-14	0/2 (0%)	0/4 (0%)	0/3 (0%)	0/1 (0%)
15-19	0/8 (0%)	1/17(6%)	1/3 (33%)	0/6 (0%)
20-29	0/25(0%)	0/44(0%)	1/16(6%)	1/47(2%)
30-39	2/38(5%)	5/55(9%)	3/20(15%)	2/29(7%)
40-49	4/19(21%)	1/49(2%)	2/15(13%)	1/31(3%)
50-59	5/47(11%)	3/42(7%)	3/25(12%)	10/41(24%)
60-69	5/42(12%)	5/36(14%)	22/52(42%)	6/41(15%)
>= 70	3/20(15%)	1/31(3%)	22/59(37%)	8/50(16%)
total	19/201(10%)	16/279(6%)	54/193(28%)	17/246(7%)

	County U		County S	
	M	F	M	F
5-9	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
10-14	0/0 (0%)	0/3 (0%)	0/0 (0%)	0/0 (0%)
15-19	0/2 (0%)	0/5 (0%)	0/5 (0%)	0/6 (0%)
20-29	0/7 (0%)	0/4 (0%)	2/19 (11%)	2/37 (5%)
30-39	1/13 (8%)	2/14 (14%)	0/22 (0%)	0/42 (0%)
40-49	1/6 (17%)	1/12 (8%)	0/10 (0%)	1/36 (3%)
50-59	2/12 (17%)	0/9 (0%)	3/24 (13%)	1/36 (3%)
60-69	0/6 (0%)	0/3 (0%)	2/30 (7%)	5/46 (11%)
>= 70	0/2 (0%)	0/1 (0%)	2/20 (10%)	2/28 (7%)
Total	4/48 (8%)	3/51 (6%)	9/130(7%)	11/231(5%)

Table 8 (cont)

Age	County Y		County W	
	M	F	M	F
5-9	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
10-14	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
15-19	0/3 (0%)	0/3 (0%)	0/0 (0%)	0/0 (0%)
20-29	2/11 (18%)	0/6 (0%)	0/1 (0%)	0/3 (0%)
30-39	1/17 (6%)	1/19 (5%)	0/4 (0%)	0/6 (0%)
40-49	1/13 (8%)	1/14 (7%)	0/5 (0%)	0/6 (0%)
50-59	4/25 (16%)	0/14 (0%)	1/4 (25%)	0/14 (0%)
60-69	8/32 (25%)	4/38 (11%)	1/8 (13%)	1/15 (7%)
>= 70	6/38 (16%)	4/56 (7%)	1/12 (8%)	1/16 (6%)
total	22/139(16%)	10/150(7%)	3/34 (9%)	2/60 (3%)

	County X	
	M	F
5-9	0/0 (0%)	0/0 (0%)
10-14	0/0 (0%)	0/0 (0%)
15-19	0/4 (0%)	0/4 (0%)
20-29	0/13 (0%)	0/12 (0%)
30-39	2/17 (12%)	1/15 (7%)
40-49	0/3 (0%)	0/12 (0%)
50-59	0/12 (0%)	1/12 (8%)
60-69	7/23 (30%)	1/33 (3%)
>= 70	5/29 (17%)	2/44 (5%)
total	14/101 (14%)	5/132 (4%)

Table 8 (cont).

	County I		County M	
	M	F	M	F
5-9	0/3 (0%)	0/1 (0%)	0/0 (0%)	0/0 (0%)
10-14	0/6 (0%)	0/3 (0%)	0/0 (0%)	0/3 (0%)
15-19	0/2 (0%)	0/0 (0%)	0/34 (0%)	0/4 (0%)
20-29	0/4 (0%)	0/7 (0%)	1/68 (2%)	0/44 (0%)
30-39	0/11 (0%)	0/6 (0%)	0/62 (0%)	0/47 (0%)
40-49	0/6 (0%)	0/5 (0%)	0/38 (0%)	1/23 (4%)
50-59	0/4 (0%)	0/8 (0%)	0/11 (0%)	0/9 (0%)
60-69	0/13 (0%)	0/19 (0%)	0/1 (0%)	0/3 (0%)
>= 70	0/43 (0%)	0/40 (0%)	0/9 (0%)	0/5 (0%)
total	0/92 (0%)	0/91 (0%)	1/223(<1%)	1/138(<1%)

Grand total.All sites together.

	M	F
5-9	0/0 (0%)	0/1 (0%)
10-14	0/5 (0%)	0/8 (0%)
15-19	1/25 (4%)	1/41 (2%)
20-29	5/92 (5%)	3/153(2%)
30-39	9/131(7%)	11/180(6%)
40-49	8/71 (11%)	5/160(3%)
50-59	18/149(12%)	15/168(9%)
60-69	45/193(23%)	22/212(10%)
>= 70	39/180(22%)	18/226(8%)
total	125/846(15%)	75/1149(7%)

Table 9.

County	Total population.	Number of clin cases 1984/85	Incidence per 1000 inhab
BD	264 457	32	0.121
AC	245 252	72	0.294
Y	264 803	55	0.208
W	285 610	20	0.070
X	291 497	19	0.065
U	256 976	2	0.008
S	281 205	14	0.050
M	745 434	0	0.000
I	55 987	0	0.000

Table 10.

Incidence of NE per year and 1000 inhabitants calculated based on clinical NE cases during a 14 year long study period.

Residents in Vindeln, Sorsele and Norsjö tested for specific anti-Puumala virus antibodies.

M = males F = females

Age	Numbers of residents		Clinical cases over 14 years		Incidence per 1000 inhab/year		Pos/sera tested(%)	
	M	F	M	F	M	F	M	F
0-9	1385	1342	0	0	0,000	0,000	0/0	0/0
10-19	1700	1599	5	0	0,210	0,000	1/6 (17%)	0/7
20-29	1229	1021	18	3	1,046	0,210	1/16 (6%)	1/47 (2%)
30-39	1102	1080	13	2	0,843	0,132	3/20(15%)	2/29 (7%)
40-49	1342	1252	11	3	0,586	0,171	2/15(13%)	1/31 (3%)
50-59	1412	1257	2	4	0,101	0,227	3/25(12%)	10/41(24%)
60	1999	1945	2	1	0,072	0,037	44/111(40%)	14/91(15%)
Total	10169	9496	51	13	2,858	0,777	54/193(28%)	28/246(11%)

Table 11

Prevalence of Puumala virus antibody and antigen among small mammals captured at various collection sites throughout Sweden, 1982-1984.

Location (yrs sampled)	*Clethrionomys glareolus* TOT	Aby	Ag	*Clethrionomys rufocanus* TOT	Aby	Ag	*Apodemus sylvaticus* TOT	Aby	Ag	*Apodemus flavicollis* TOT	Aby	Ag	*Rattus norvegicus* TOT	Aby	Ag	*Sorex* sp. TOT	Aby	Ag	Other Species* TOT	Aby	Ag	Total TOT	Aby	Ag
North of Limes Norrlandicus																								
Abisko (82)	0	0	0	105	1 (1%)	0	0	0	0	0	0	0	0	0	0	23	NT**	0	0	0	0	128	1	0
Kiruna (82)	0	0	0	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0
Vindeln (82-84)	161	27 (17%)	16 (10%)	3	0	0	2	0	0	0	0	0	0	0	0	23	NT	0	5	0	0	194	27	16
Sundsvall (84)	34	12 (35%)	6 (18%)	0	0	0	0	0	0	1	1	0	0	0	0	6	NT	0	1	0	0	42	13	6
Arvika (83-84)	81	2**	0	0	0	0	10	0	0	3	0	0	0	0	0	5	NT	0	2	0	0	101	2	0
SUBTOTAL	276	41 (15%)	22 (8%)	125	1 (<1%)	0	12	0	0	4	1 (25%)	0	0	0	0	57	NT	0	8	0	0	482	43 (9%)	22 (5%)
South of Limes Norrlandicus																								
Revinge (82-84)	131	1 (<1%)	0	0	0	0	52	0	0	4	0	0	0	0	0	1	NT	0	0	0	0	188	1	0
Gotland (82)	0	0	0	0	0	0	0	0	0	0	0	0	2	NT	0	0	0	0	3	NT	0	5	NT	0
Malmo	0	0	0	0	0	0	0	0	0	0	0	0	6	1	0	0	0	0	1	0	0	7	1	0
SUBTOTAL	131	1 (<1%)	0	0	0	0	52	0	0	4	0	0	8	1	0	1	NT	0	4	0	0	200	2	0
OVERALL TOTAL	407	42 (10%)	22 (5%)	125	1 (<1%)	0	64	0	0	8	1 (13%)	0	8	1 (13%)	0	58	NT	0	12	0	0	682	45 (7%)	22 (3%)

*Includes 8 *Mus. Musculus*, 1 *Microtus agrestis*, 3 *Myopus schisticolor*.
**NT = not tested.

Table 12.

Different species of rodents infected with PUU virus (lung pool material from infected C. glareolus and tested 60 days later for presence of PUU specific antibodies in serum and PUU specific antigen in lungs. Mus Musculus, Apodemus flavicollis, C.rutilus and C.rufocanus all originated from endemic areas. C.glareolus (Stöm) originated from western parts of endemic areas while C.glareolus (Ammar) originated from the eastern parts of endemic areas. C.glareolus (Lund) originated from the southern tip of Sweden wich is non-endemic.

Species	Number of animals	Antibody positive	Antigen positive	Antigen and antibody positive
Mus Musculus	10	0	0	0
Apodemus flavicollis	10	0	0	0
C.glareolus (Stöm)	21	11 (52%)	7 (33%)	7
C.glareolus (Lund)	39	20 (51%)	10 (26%)	9
C.glareolus (Ammar)	51	37 (72%)	33 (65%)	32
C.rutilus	6	2 (33%)	1 (17%)	1
C.rufocanus	16	9 (56%)	1 (6%)	1

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Figure 1.

Acute and/or early convalescent and 3-9 months convalescent sera from Swedish patients with clinical NE tested by IgM ELISA. Sera from the same patient are conected with a line.

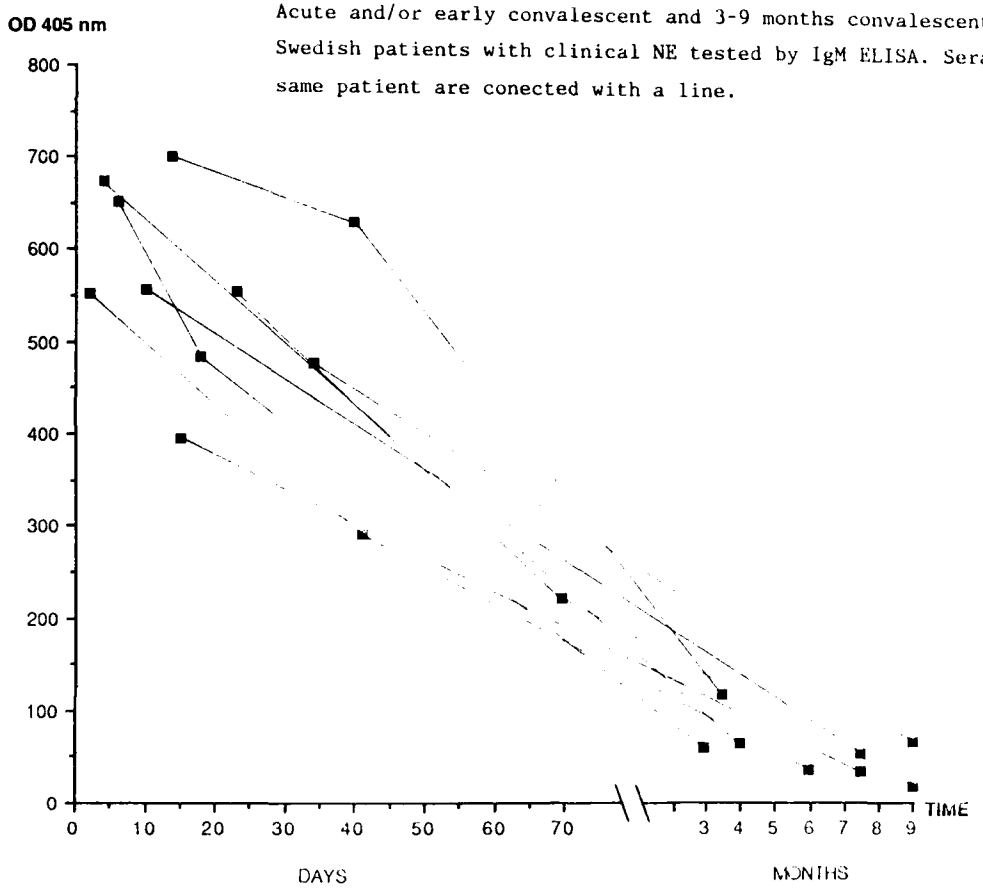


Figure 2.
Acute and/or early convalescent and 2 years convalescent sera from Swedish patients with clinical NE tested by IgM ELISA. Sera from the same patient are conected with a line.

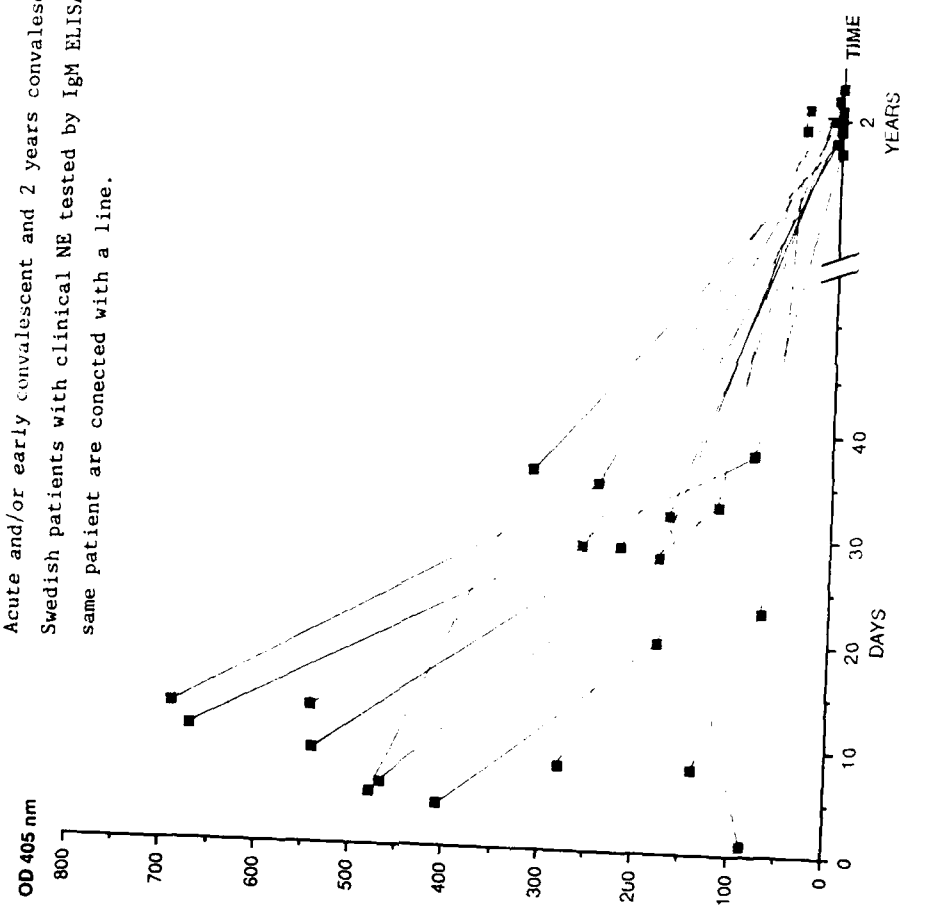


Figure 3.

Acute (day 1-9), early convalescent (day 16-42) and late convalescent sera (2 year) (n=49) from 21 Swedish patients tested by IgG ELISA and IgG IFT. Correlation coefficient 0.71, $p < 0.01$, slope 0.27, standard error of the slope 0.04.

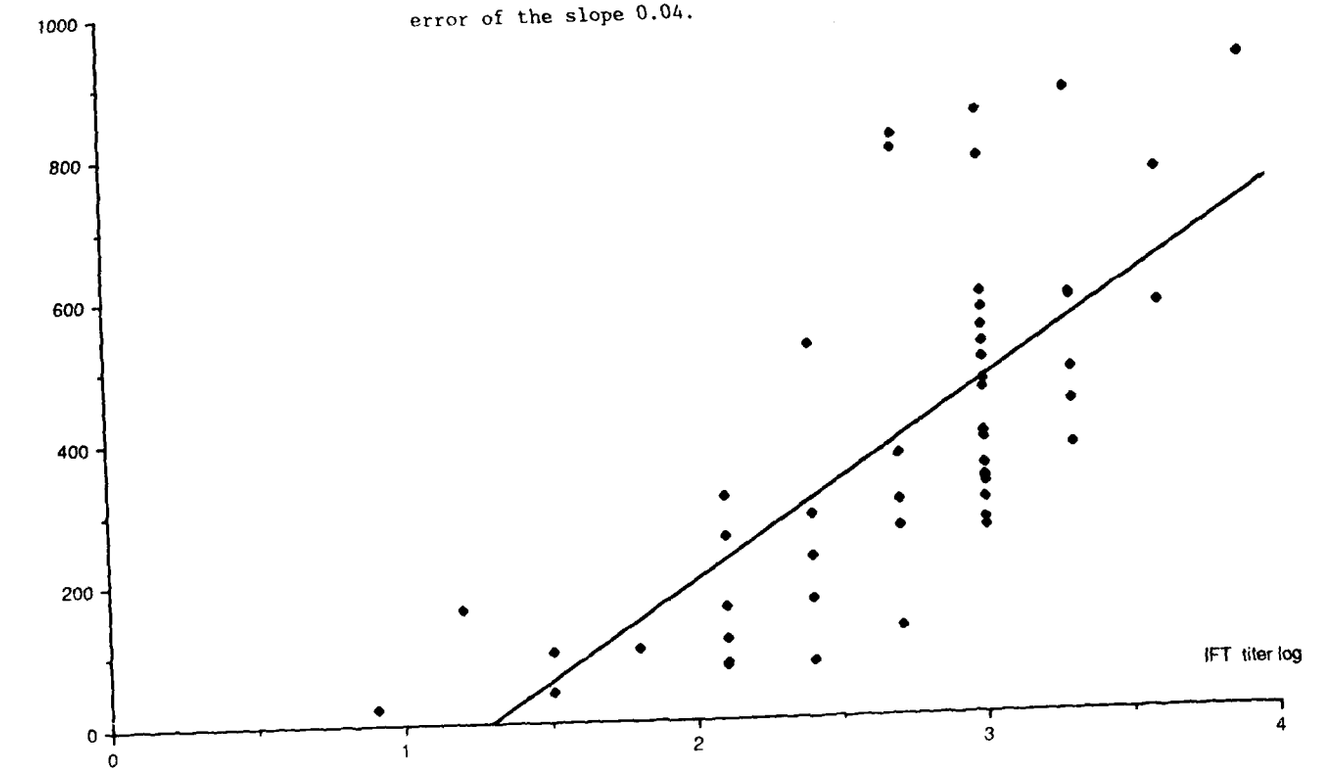


Figure 4.

Acute (day 1-9), early convalescent (day 16-42) and late convalescent sera (2 year) (n=49) from 21 Swedish patients tested by IgG ELISA. Each sera are plotted as well as the mean of acute, early convalescent and late convalescent sera.

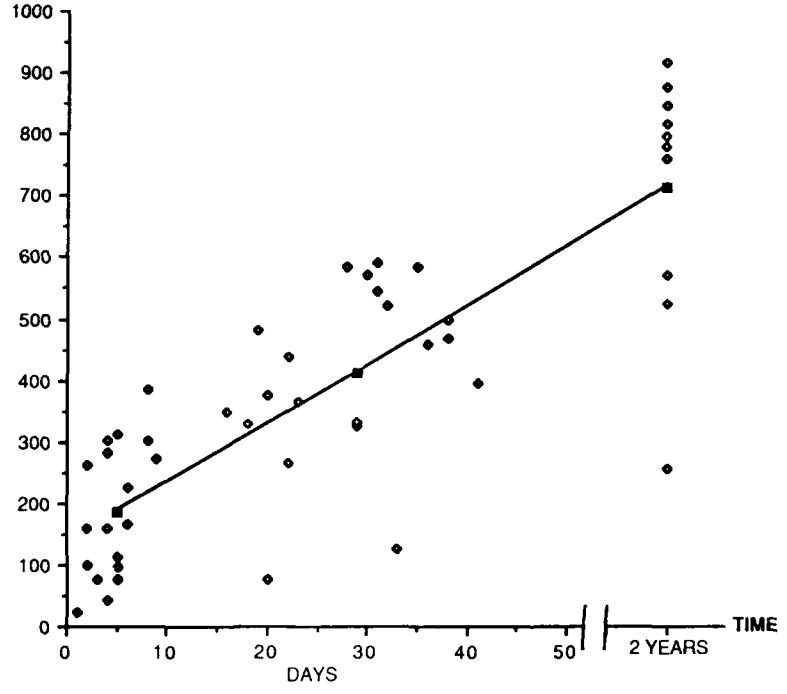


Figure 5.

Acute (day 1-9), early convalescent (day 16-42) and late convalescent sera (2 year) (n=49) from 21 Swedish patients tested by IgG IFT. Each sera are plotted as well as the geometric mean of acute, early convalescent and late convalescent sera.

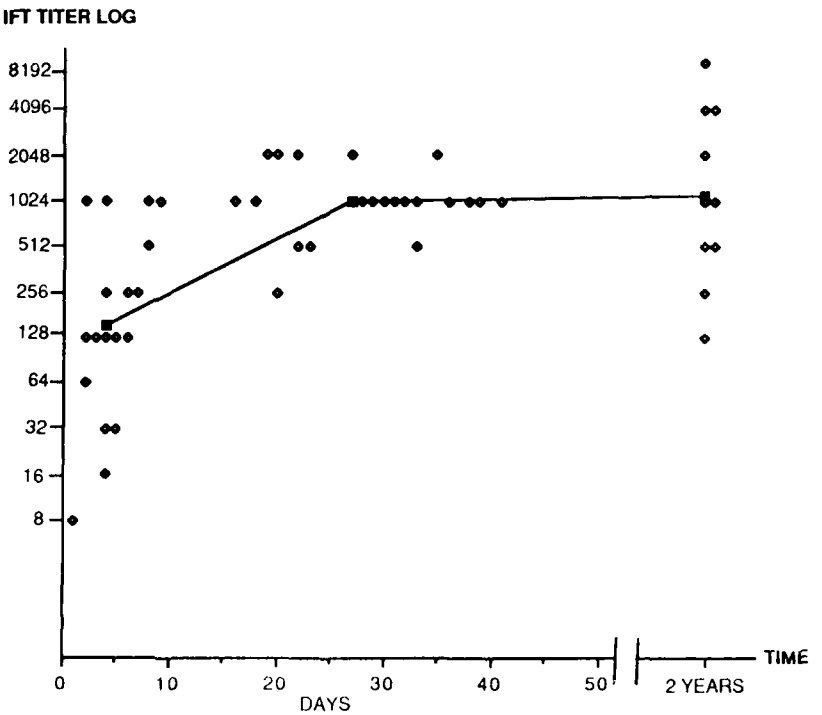
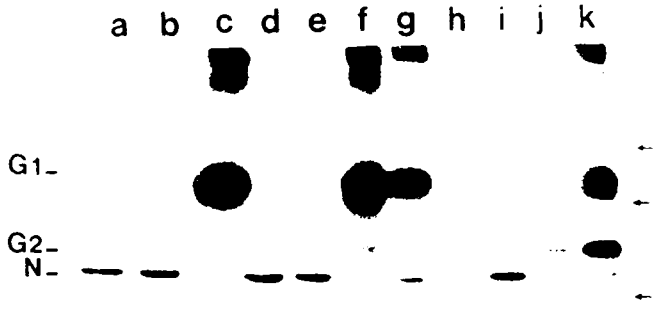
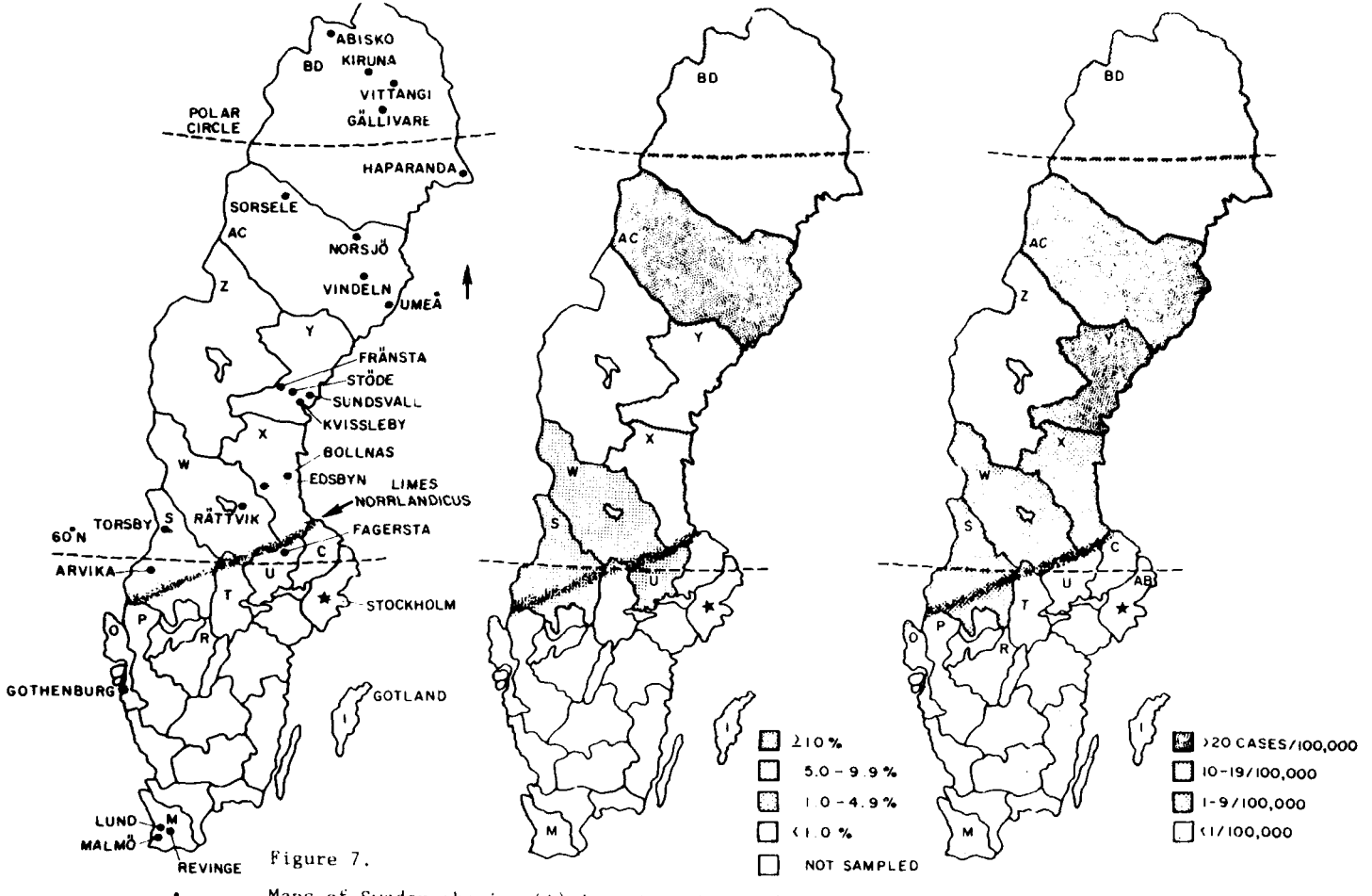


Figure 6.

Reactivity of the Urban rat (80-39) virus proteins with HFRS patient and rabbit immune sera in RIPA. 35-S-methionine radiolabelled virus antigen was immunoprecipitated with the following sera:
Asian U.S.S.R patients'sera 27-2 and 83-3 (lanes a and b respectively);
European U.S.S.R. patients' sera T and 3258 (lanes c and d respectively);
Korean patients'serum 700047 (lane e);
Swedish patients'sera M and RG (lanes f and g respectively);
HFRS negative human serum (lane h);
Rabbit anti Hantaan 76-118 (lane i);
Rabbit anti Puumala 83-223L (lane j);
Rabbit anti Urban rat 80-39 (lane k).
Arrowheads indicate the position of mol. wt markers: phosphorylase b, 92500; bovine serum albumine, 69000; ovalbumin, 46000; carbonic anhydrase, 30000; and lactoglobulin, 18400.





A

Figure 7.

Maps of Sweden showing (A) locations (cities) where serosurvey samples were obtained and where small mammal collections were made during 1982-1985, (B) age-adjusted prevalence rates of antibody to Puumala virus by county; AC, Västerbotten county; Y, Västernorrland county; X, Gävleborg county; W, Kopparberg county; S, Värmland county; U, Västmanland county; M, Malmö county; I, Gotland county (island), and Z, Jämtland county.

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